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Development of anti-EGF receptor peptidomimetics (AERP) as tumor imaging agent

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ABSTRACT

EGFR is over-expressed in several solid tumors including breast, prostate, pancreas, and lung cancers and is correlated to the metastasic potential of the tumor. Anti-EGFR receptor-binding peptidomimetics (AERP) were examined to assess the small molecule's potential use as tumor-specific imaging agents. The aim of this work was to design and characterize the binding specificity of the radiolabeled peptidomimetics to EGFR over-expressing cell lysate and to A431 xenograft tumors. Our newly designed peptidomimetic, AERP, was conjugated to DTPA and labeled with ^{99m}Tc. The in vivo tumor accumulation of [^{99m}Tc] DTPA-AERP-2 was 1.6 ± 0.1 %ID/g and tumor to muscle ratio was 5.5. Our studies suggest that this novel peptidomimetic, AERP-2, warrants further development as an EGFR specific tumor-imaging agent.

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The epidermal growth factor (EGF) family of tyrosine kinase receptors was expressed at high levels in a wide variety of human cancers and had been associated with various features of advanced disease and poor prognosis. The EGFR tyrosine kinases are found to be involved in proliferation, signal transduction, angiogenesis and apoptosis of cancer cells.[1–3] The tyrosine kinase family receptors are over-expressed in 20–30% cancers of the breast,[4] prostate,[5] ovary,[6] and pancreas.[7] The over-expressed receptors promote the growth and metastasis of cells.[8]

Monoclonal antibodies have already shown promise in targeting epidermal growth factor receptor-positive tumors.[9,10] There are various drugs in the market such as Iressa,[11] which are used as anticancer drugs and targets EGFR. Another drug in clinical trial is Tarceva.[12] Molecules such as IMC-C225 (anti-EGFR antibody) can specifically target the receptors on cancer cells to interrupt the transduction of growth signals,[13] prohibit metastasis,[14] and induce apoptosis[15] of the cancer cells. Because of their receptor-binding specificity, IMC-C225 could be used as imaging/therapeutic agents for tumors when radiolabeled.[16]

Epidermal growth factor (EGF) is a ~6 KDa polypeptide and first ligand known to bind EGFR. Because of its high affinity to EGFR, investigators radiolabeled EGF and exploit its potential in tumor targeting.[17,18] Since it is a natural ligand, using EGF as probe carry risk of activating EGFR receptors, and thereby potentiate tumorigenesis. We believe molecules that show anti-tumor effects might be a better candidate for tumor imaging studies. Antibodies are high affinity molecules that show anti-tumor effects. However, the radiolabeled antibodies, despite the high percentage of injected dose accumulated in tumors after a few days circulation, exhibited slow clearance from blood and therefore caused high background for imaging and excessive toxicity to normal organs in therapy. Smaller molecules with inherent receptor-binding affinity would overcome these problems because clearance of the molecules from the circulation is faster compared to the monoclonal antibody. Recently investigators generated a fully human anti-EGFR Fab that recognizes the extra-cellular domain of EGFR and showed labeled anti-EGFR Fab had reasonable antigen-binding capability and accumulated only in tumors with high or moderate EGFR expression.[19] These and many other investigators employed various other antibody fragments such as nanobody,[20] single domain antibody format,[21] affibody[22] but many of these results are still in very early stage

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of their research and its not clear if they can be utilized clinically in mapping EGFR receptors.

In recent years, radiolabeled peptides have been successfully developed and used in the clinic for nuclear medicine receptor imaging.[23,24] In addition, several radiolabeled peptides, such as [^{99m}Tc] neurotensin (NT) for pancreatic tumor imaging,[25] bombesin analogues with ^{99m}Tc-labeled for tumor imaging,[26] and [^{64}Cu]RGD for $\alpha_V\beta_3$ integrin imaging,[27] have shown promising results.

In this preliminary work, a radiolabeled peptidomimetic (AERP) was evaluated as a tumor-specific agent. In order to design EGFR specific peptidomimetics, we used the deduced structure of the monoclonals (MAb) 225 and 425 resolved by X-ray crystallography (Murali, unpublished). A bioactive anti-EGF receptor peptidomimetic (AERP) has been designed from the three-dimensional information of two anti-EGFR monoclonal antibodies. The newly fashioned AERP mimetics bind to the EGFR, with low nanomolar affinity. This interaction has been demonstrated to impart biological function similar to the interaction of a full-length monoclonal antibody raised against this growth receptor oncoprotein. Two AERP analogs have been resolved. The sequence of the peptide is shown in Figure 1. A control peptide CD4 ([Cys^3-Cys^{12}]-H-Abu-FCYIGEVEDQCY-OH) was synthesized in our lab. These peptides were cyclized, purified by HPLC.

Suitable peptidomimetics for solution structure by NMR were selected by performing one-dimensional NMR spectra under varying salt, pH and temperature conditions. The optimum conditions for structural characterization of these peptides were found to be in 50 mM acetate buffer, with 100 mM NaCl at pH 4.5 and 14 °C. A pair of 2-dimensional experiments were used to connect protons within a given spin system and determine their sequential assignments. The NOESY and TOCSY spectra for the peptides were collected on a 750 MHz spectrometer. Structural constraints for chemical shift, dihedral angles and through space distances were collected and the structure calculations were performed with the CNS program[28] using the NMR constraints as inputs. A simulated annealing protocol was used to generate a family of structures that satisfied the 217 distance and 10 dihedral experimental constraints. The structure refined to 0.6 Å pair-wise RMSD for the backbone atoms for a set of 17 structures.

The structures of the 15 residue (Fig. 2) AERP-2 peptide analogs contain a bend at the N-terminal portion fixed by a disulfide linkage between residues 2 and 8. The middle section contains a 3–10 helix and the C-terminal end is disordered. Three of the four aromatic side chains are clustered on one side of the molecule. A common design feature of the peptides is the positioning of two cysteine residues that constrain the structures. Some peptides produced aggregate states through the possible formation of intermolecular disulfide bonds. Other peptides containing proline residues displayed multiple cis/trans isomeric states. The resulting structure of the exocyclic peptide revealed features not predicted by the molecular modeling applied to this peptide such as residues that might influence aggregates and isomerization.

We obtained baculovirus-produced and partially purified ectodomain forms of EGFR species from Mark Lemmon (University of Pennsylvania). The ectodomain construct was further purified by high performance gel filtration chromatography. Kinetic binding characteristics of AERP to the ectodomain of the EGF receptor were studied using biosensor techniques.[29,30] AERP binds to the EGF receptor with an approximate affinity of 400 nM. At optimum surface density (3600 RU), AERP binds to EGF receptors in a concentration-dependent manner with a dissociation pattern (k_{off}) within an order of magnitude to that of the 225 MAb (data not shown). Importantly, AERP did not show binding to either immobilized Her2/neu or TNF receptors.

AERP-2 (Primary structure: dYCASRDYDYDGRCYFD-NH2)

AERP-2A (Primary Structure: YCRDYDYDGRYFDCY-NH2)

Figure 1. Amino acid sequences of two derivatives of AERP.

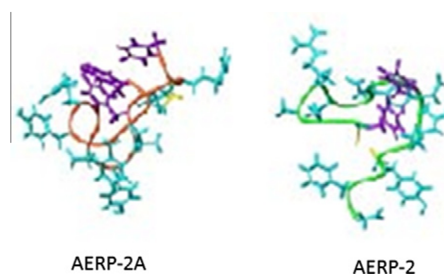

Figure 2. Solution structure of AERP mimetic analog. The overall fold between AERP-2 and AERP-2A is similar (RMSD for back bone structure is 2.9 Å) and RMSD for all non-hydrogen atoms is 5.64 Å. The disposition of Arg residues in these two structures is different and the results show the effect of introducing a serine in the cyclic ring of AERP.

The intramolecular cyclization of the peptides (0.1 mg/mL or 0.5–0.7 mM, pH 8–8.5) was carried out at 4 °C as described previously.[29,30] The advance of the cyclization process was monitored by testing the concentration of the free thiol groups of the peptide by the DTNB method.[29,30] The testing procedures were: (a) 400 μL of the reaction solution was mixed with 100 μL of 0.1 M pH 8 phosphate buffer and 5 μL of 10 mM pH 8 of DTNB; (b) the mixture was incubated at 4 °C in the dark for 30 min; (c) absorption at 412 nm of the mixture was measured with the reaction solution as reference; (d) cyclization was considered to be completed when the absorptions at 412 nm of the mixture and the reaction solution became identical. After the completion of the cyclization, the solvents were removed by lyophilization. The purity of the cyclized peptides was tested by HPLC (ODS C18 column, 0.01 M pH 5.2 NH_4OAc/MeOH as mobile phase with gradient of 40% MeOH to 90% MeOH over 25 min) before further use.

For imaging purposes, AERP was conjugated to DTPA (Fig. 3) in order to do conjugate the cyclic peptide to DTPA, 10 mg/mL solution of the cyclized peptide in water was added an equal volume of 0.2 mol/L bicarbonate buffer, pH 8.6. A suspension of the DTPA cyclic anhydride in DMSO (10 mg/mL) was then added drop-wise with agitation to achieve a final DTPA-to-peptide molar ratio of 3:1. The mixture was stirred at room temperature for 1 h and the DTPA conjugate was purified by HPLC employing 0.01 M NH_4OAc/MeOH as mobile phase. HPLC purified product containing solvent was evaporated by rotary evaporation at 35 °C and lyoph-

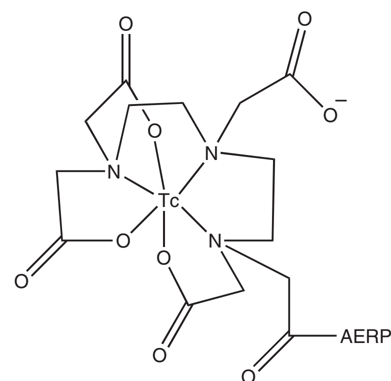

Figure 3. The schematic structure of AERP–DTPA–Tc complex.

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